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# Neurons and Ecdysteroids Promote the Proliferation of Myogenic Cells Cultured from the Developing



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**During metamorphosis in the hawkmoth *Manduca sexta*, larval leg motoneurons survive the degeneration of their target muscles to innervate new muscles that form during the development of the adult legs. Observations of muscle development *in vivo* suggest that there are close interactions between motor terminals and the muscle precursor cells at the earliest stages of muscle formation and surgical denervation compromises further development of adult muscles. Here we describe a nerve/muscle coculture system that allows further exploration of this critical developmental interaction. Muscle precursor cells derived from the developing thoracic legs of early pupae and cultured in the presence of neurons assumed a spindle-like morphology and fused to form multinucleate contractile myotubes. Contractile fibers did not form in cultures of muscle precursor cells alone. In the presence of neurons the rate of bromodeoxyuridine (BrdU) incorporation into myonuclei was significantly enhanced, suggesting that neurons promote the proliferation of myogenic cells. This effect was not unique to thoracic leg motoneurons of the early pupal stage, in that larval thoracic neurons as well as neurons from the pupal brain or abdominal ganglia were also effective at enhancing BrdU incorporation and the formation of contractile muscle fibers. Medium conditioned by neurons was ineffective at promoting BrdU incorporation, and in cocultures BrdU incorporation was enhanced only in regions of physical overlap between neurons and muscle precursor cells, suggesting that a very close-range interaction was involved. Tetrodotoxin-sensitive neuronal activity was not required for the effect on muscle development, but fixed neurons were ineffective. The insect steroid hormone 20-hydroxyecdysone enhanced BrdU incorporation into the nuclei of myogenic cells in both the presence and the absence of neurons. The results suggest that both neurons and ecdysteroids play an important regulatory role in adult muscle development, at least in part by enhancing the proliferation of myogenic cells.** © 1996 Academic Press, Inc.

## INTRODUCTION

Developing neuromuscular systems have long served as useful models for investigating the importance of cellular interactions during development. In vertebrate systems myoblast proliferation and the differentiation of muscle fiber type are independent of innervation at early stages, but secondary myoblasts are influenced by cues provided by motoneurons (Donoghue and Sanes, 1994; Harris, 1981; Stockdale, 1992; Miller and Stockdale, 1987; Ross *et al.*, 1987). Further differentiation of muscle fibers, including the synthesis and localization of acetylcholine receptors, is regulated by the neurons through increasingly well-characterized cellular and molecular mechanisms (Hall and Sanes, 1993).

In insect systems, the development of neuromuscular systems occurs in two phases. During embryonic development, the larval muscles form from mesodermal derivatives that proliferate, fuse, and differentiate prior to innervation (Ball *et al.*, 1985; Ball and Goodman, 1985a,b; Bate, 1990; Broadie and Bate, 1993a; Johansen *et al.*, 1989). Neuronal interactions are not essential for most aspects of larval muscle development (Broadie and Bate, 1993b), although the normal synthetic rate and localization of glutamate receptors are dependent upon innervation (Broadie and Bate, 1993c,d).

During the postembryonic development of holometabolous insects, such as *Drosophila* and the moth *Manduca sexta*, many larval muscles degenerate at the onset of metamorphosis. New adult muscles form from myoblasts that proliferate, fuse, and differentiate during metamorphosis (Bate *et al.*, 1991; Currie and Bate, 1991; Fernandes *et al.*, 1991; Fernandes and VijayRaghavan, 1993; Kent *et al.*, 1995; C. Consoulas, K. S. Kent, M. Anezaki, and R. B. Levine, submitted for publication). In *Manduca* these imaginal muscles are innervated by embryonically derived motoneurons that persist following the degeneration of their larval targets (Levine and Truman, 1985; Kent and Levine, 1988), and the same is probably true of many imaginal muscles in *Drosophila* (Fernandes and VijayRaghavan, 1993; Hummon and Cos-

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tello, 1987). The imaginal muscles develop in close association with the distal processes of motoneurons (Fernandes and VijayRaghavan, 1993; Currie and Bate, 1991; Truman and Reiss, 1995; C. Consoulas, K. S. Kent, and R. B. Levine, submitted for publication), and several lines of evidence suggest that, in contrast to the embryonic development of larval muscles, neuronal interactions are necessary for the development of these adult muscles. Thus, the development of adult flight muscles in silkmoths is compromised if innervation is interrupted prior to the onset of metamorphosis (Nüesch, 1985). Similarly, muscles of the adult thoracic legs in *Manduca* do not develop normally if innervation of the leg is interrupted prior to the onset of metamorphosis (C. Consoulas and R. B. Levine, unpublished observations), and denervation prevents the normal development of adult abdominal muscles (Thorn and Truman, 1989; Hegstrom and Truman, 1996; R. J. Bayline, A. B. Khoo, and R. Booker, submitted for publication). In *Drosophila* gynandromorphs the development of an adult male-specific abdominal muscle requires a male nervous system (Lawrence and Johnston, 1986), and the critical requirement for innervation has been confirmed through surgical manipulations (Currie and Bate, 1995).

Does the neuronal influence on imaginal muscle development represent an effect on the proliferation of myoblasts, their accumulation and subsequent fusion and differentiation into muscle fibers, or their maintenance? In order to address this question, we have developed a nerve/muscle coculture system that allows us to explore the mechanisms underlying nerve/muscle interactions. We find that the neuronal dependence of muscle development is recapitulated in this culture model.

## MATERIALS AND METHODS

### *Animals*

*M. sexta* (Lepidoptera: Sphingidae) were reared from eggs on artificial diet (modified from Bell and Joachim, 1976) on a long-day photoperiod regimen (17 hr light/7 hr dark at 26°C and 50–60% relative humidity. Following hatching, larvae feed continually and pass through five larval instars. Near the end of the last (fifth) larval instar, in response to changes in the ecdysteroid and juvenile hormone titers, animals begin metamorphosis. Adult development begins soon after the molt of the larva into the pupa and proceeds through 18 stages, corresponding roughly to days (P0–P18). Pupae were staged as previously described (Sanes and Hildebrand, 1976; Tolbert *et al.*, 1983).

### *Preparation of Neuronal Cultures*

The thoracic ganglia were dissociated and the neurons maintained in culture using a modification of techniques that have been described previously (Hayashi and Levine, 1992; Prugh *et al.*, 1992). Animals were dissected at stage P0 (the first day of the pupal stage). The three thoracic ganglia from each animal were removed in sterile insect saline (see below) and pinned in a petri dish lined with Sylgard (Dow Corning). The ganglia were desheathed and separated from the interganglionic connectives and peripheral nerves in the same saline. The individual desheathed ganglia were then transferred to a test tube containing supplemented saline (see below). For these high-density neuronal cultures enough thoracic ganglia were pooled to yield an average of three per dish.

The following procedures were performed in a hood under aseptic conditions. The ganglia to be dissociated were transferred to a tube with Hanks $Ca^{2+}$- and $Mg^{2+}$-free balanced salt solution containing 0.1 mg/ml collagenase (Worthington) and 0.4 mg/ml dispase (Boehringer-Mannheim), incubated for 6 min at 37°C, and then dispersed by trituration with a fire-polished Pasteur pipette. The action of the enzymes was terminated by centrifuging the cells first through 6 ml of supplemented saline, then resuspending the pellet and centrifuging through 6 ml of modified L-15 medium (see below). The pellet was resuspended in sufficient medium to allow 100 $\mu$l for each culture dish. The cultures were maintained at 26°C in a humidified incubator.

Cells were grown in miniwells made by cutting an 8-mm hole in the bottom of a plastic 35-mm culture dish. Glass coverslips were sealed to the bottom of the dishes with Sylgard. After UV sterilization, the glass was coated by exposure to a solution of 200 $\mu$g/ml Concanavalin A (Sigma) and 2 $\mu$g/ml laminin (Collaborative Research) for 2 hr at 37°C. The dishes were then rinsed with sterile distilled water and air-dried in a sterile hood.

The contents of the solutions used were derived from Hayashi and Hildebrand (1990).

***Insect saline.*** NaCl, 100 m*M;* KCl, 4 m*M;* $CaCl_2$, 6 m*M;* Hepes, 10 m*M*, pH 7; and Glucose, 5 m*M;* adjusted to 360 mosM with mannitol.

***Supplemented saline.*** NaCl, 149.9 m*M;* KCl, 3 m*M;* $CaCl_2$, 3 m*M;* $MgCl_2$, 0.5 m*M;* TES 10 m*M;* D-glucose, 11 m*M;* lactalbumin hydrolysate, 6.5 g/liter; TC Yeastolate (Difco) 5 g/liter; 10% fetal bovine serum (FBS); penicillin, 100 units/ml; streptomycin, 100 $\mu$g/ml, pH 7; 360 mosM.

***Modified L-15 culture medium.*** To 500 ml of Leibovitz's L-15 (Gibco) was added: $\alpha$-ketoglutaric acid, 185 mg; fructose, 200 mg; glucose, 350 mg; malic acid, 335 mg; succinic acid, 30 mg; TC yeastolate, 1.4 gm; lactalbumin hydrolysate, 1.4 gm; niacin, 0.01 mg; imidazole, 30 mg; streptomycin, 100 $\mu$g/ml; penicillin, 100 units/ml; 20-hydroxyecdysone, 1 $\mu$g/ml (Sigma); 10% FBS; and stable vitamin mix, 2.5 ml. A 5-ml stock solution of vitamin mix consisted of: aspartic acid, 15 mg; cystine, 15 mg; $\beta$-alanine, 5 mg; biotin, 0.02 mg; vitamin $B_{12}$, 2 mg; inositol, 10 mg; choline chloride, 10 mg; lipoic acid, 0.5 mg; *p*-aminobenzoic acid, 5 mg; fumaric acid, 25 mg; coenzyme A, 0.4 mg; glutamic acid, 15 mg; phenol red, 0.5 mg. The medium was adjusted to pH 7, and 360 mosM. Both the supplemented saline and the medium were filter-sterilized prior to use.

### *Preparation of Muscle Cell Cultures and Cocultures*

To obtain cultures of muscle precursor cells, early pupae (stage P2) were surface-sterilized with 70% ethanol, then

the developing legs were removed from the pupal cuticle and placed in supplemented saline solution. The legs were pinned in a small petri dish containing insect saline, cut open length-wise, and then placed in a tube containing Hank's balanced salt solution including 0.1 mg/ml collagenase and 0.4 mg/ml dispase. The internal tissue was dissociated with gentle trituration after incubation for 6 min at 37°C, and the resulting cell suspension was added to a tube containing supplemented saline and centrifuged (250 rpm/3 min) to remove large debris. The supernatant was then transferred to a clean tube and centrifuged again at 1000 rpm for 3 min, after which the pellet was resuspended in L-15/Grace's medium (see below), and centrifuged again. The pellet was resuspended again in sufficient L-15/Grace's medium to yield 100 $\mu$l of suspension for each culture dish. In these experiments an average of one thoracic leg was used per culture dish. The density of live cells in the suspension was determined with a hemocytometer after mixing a 25-$\mu$l aliquot to 25 $\mu$l of 0.25% trypan blue in PBS. Typical densities were $3–5 \times 10^4$ cells/100 $\mu$l.

The L-15/Grace's medium was prepared by supplementing the L-15-based medium described above with 20% Grace's insect medium that had been modified according to recommendations provided by Dr. Dwight Lynn for the culture of an embryonic cell line from *Manduca* (Hayashi and Hildebrand, 1990). To 1000 ml Grace's insect medium (Gibco) was added: 350 mg $NaHC0_3$, 3 g TC yeastolate, 3 g lactalbumin hydrolysate and 100 ml FBS, pH 6.2.

For cultures of leg cells alone, 100 $\mu$l of cell suspension was plated onto glass coverslips coated as above. For cocultures with neurons, as much medium as possible was removed from 2-day-old neuronal cultures and 100 $\mu$l of leg cell suspension was seeded onto the dishes. The dishes were sealed with parafilm and incubated at 26°C. After 2 days each dish was flooded with 1 ml of the L-15/Grace's medium. For all experiments the leg cell suspension was divided among empty culture dishes and dishes into which neurons had been plated 2 days previously. All comparisons were, therefore, performed between paired cultures that had been prepared together and had the same original density of leg cells.

For experiments in which the role of 20-hydroxyecdysone (20-HE) was examined, medium was prepared without adding 20-HE. This medium was divided and an appropriate level of 20-HE was added to half (final concentration, 1 $\mu$g/ml). Thus, all experiments involved comparisons among cultures prepared with the same batch of medium.

### *Cell Proliferation Assay*

5-Bromodeoxyuridine (BrdU) incorporation was used to identify cells undergoing DNA synthesis *in vitro*. For the desired time interval the culture medium was replaced with medium containing 20 $\mu M$ BrdU (Sigma). At the desired time the cultures were fixed in acidified alcohol (30 min; 7 parts 100% ethanol to 3 parts acetic acid). To block endogenous peroxidase activity, the cultures were dehydrated through an ethanol series into methanol, then incubated for 15 min in methanol containing 0.3% $H_2O_2$ and rehydrated. After rinsing in PBS (50 ml 0.2 *M* phosphate buffer, 1 liter $dH_2O$, 9 g NaCl; pH 7.4) the cultures were incubated for 15 min in 2 *N* HCl in PBS, then rinsed in 10 m*M* PBS containing 0.1% Triton X-100 (PBS-X). The cultures were then exposed to primary antibody (anti-BrdU; Becton–Dickinson) diluted 1:50 in PBS for 2 hr 15 min at room temperature, rinsed in PBS-X, then incubated for 2 hr in secondary antibody diluted 1:1000 in PBS (goat anti-mouse IgG peroxidase conjugate, Jackson Immunochemical). The cultures were next rinsed in PBS-X, PBS, and acetate–imidazole buffer (77.5 ml $dH_2O$, 17.5 ml 1 *M* sodium acetate, 5 ml 0.2 *M* imidazole, adjusted to pH 7.4 with glacial acetic acid). For the DAB reaction the chromogen solution included 8.25 ml $dH_2O$, 1.25 ml 1 *M* sodium acetate, 0.5 ml 0.2 *M* imidazole, 0.26 g $NiSO_4$ (FLUKA), and 2.5 mg DAB. Just before adding to the culture dishes, 3 $\mu$l of 30% $H_2O_2$ was added to the solution. After 5 min the cultures were rinsed in acetate–imidazole buffer, followed by PBS. Cultures were maintained in PBS for viewing or the coverslips were removed and mounted on glass slides after dehydration and clearing. Labeled nuclei were counted using phase-contrast or Hoffmann differential interference contrast microscopy on a Nikon Diaphot inverted microscope. Unless otherwise specified all labeled nuclei within each dish were counted.

### *Assessment of Cell Viability*

Cell viability was assessed using the LIVE/DEAD kit (Molecular Probes), using the manufacturer's recommended protocol. Dead cells are revealed with ethidium homodimer, which enters damaged cells and produces a bright red fluorescence upon binding to nucleic acids. Live cells are stained with calcein AM, which enters cells and is converted by esterases into calcein to produce a green fluorescent signal that is retained within live cells.

### *Phalloidin Staining*

Cocultures were stained with rhodamine–phalloidin (Molecular Probes) to label filamentous actin, using a protocol recommended by the manufacturer.

## RESULTS

### *Development of Nerve/Muscle Cocultures*

To obtain cocultures containing neurons and muscle precursor cells, stage P0 thoracic ganglia were dissociated and the neurons maintained in a 100-$\mu$l drop of modified L-15 medium. Two days later, a suspension of cells isolated from stage P2 developing adult thoracic legs was seeded on top of the neurons, which by that time had begun to extend processes and were the only cell type remaining from the dissociated thoracic ganglia (Prugh *et al.*, 1992). The cocultures were maintained thereafter in a mixture of the modified L-15 medium and Grace's medium (see Materials and

Methods). Stage P2 legs (roughly 2 days into adult development) were chosen as a source of muscle precursors because this stage marks the beginning of the major wave of myoblast proliferation that will generate the adult leg muscles (C. Consoulas, K. S. Kent, M. Anezaki, and R. B. Levine, submitted for publication).

Shortly after seeding the leg cell suspension there was a relatively uniform dispersion of small round phase-bright cells over the surface of the culture dishes. Over the first 48 hr *in vitro* there was a progressive migration of these cells into aggregates as viewed with time-lapse video microscopy or sequential photographs (Fig. 1). By day 2 *in vitro* phase-bright spindle-shaped cells appeared on the circumference of the aggregates of small round cells. In addition, a small number of flat polygonal-shaped cells with large nuclei emerged from the aggregates. The spindle-shaped cells increased in numbers dramatically (see below), and by Day 4 were aligning and fusing to form multinucleate contractile fibers (Figs. 1 and 2). Because they were multinucleate, able to contract, and displayed intense staining with rhodamine–phalloidin (not shown), we have classified these fibers as myotubes and concluded that the spindle-shaped cells that fuse to produce them are muscle precursors (myocytes). We will consider below whether the spindle-shaped cells remain capable of replication (i.e., may be termed ''myoblasts'').

The myotubes were often anchored at both ends to the cell aggregates (Figs. 1 and 2), which were pulled together as the myotubes contracted, leaving areas of the dish surface devoid of cells. Contraction of the myotubes that were anchored firmly continued for several months (up to 13 months in cultures that were allowed to survive), although the neurons survived no longer than 2 months.

In the absence of neurons, cells from stage P2 legs migrated to form aggregates and flat polygonal-shaped cells appeared in the spaces between aggregates as described above (Fig. 1). Spindle-shaped cells were also present, but in lower numbers than in the presence of neurons. Fusion of these cells was sometimes observed, but the density of spindle-shaped cells was so low that this rarely occurred. Thus, the formation of contractile myotubes was never observed in the absence of neurons, suggesting that neurons promoted either the survival, proliferation, or differentiation of the myogenic cells.

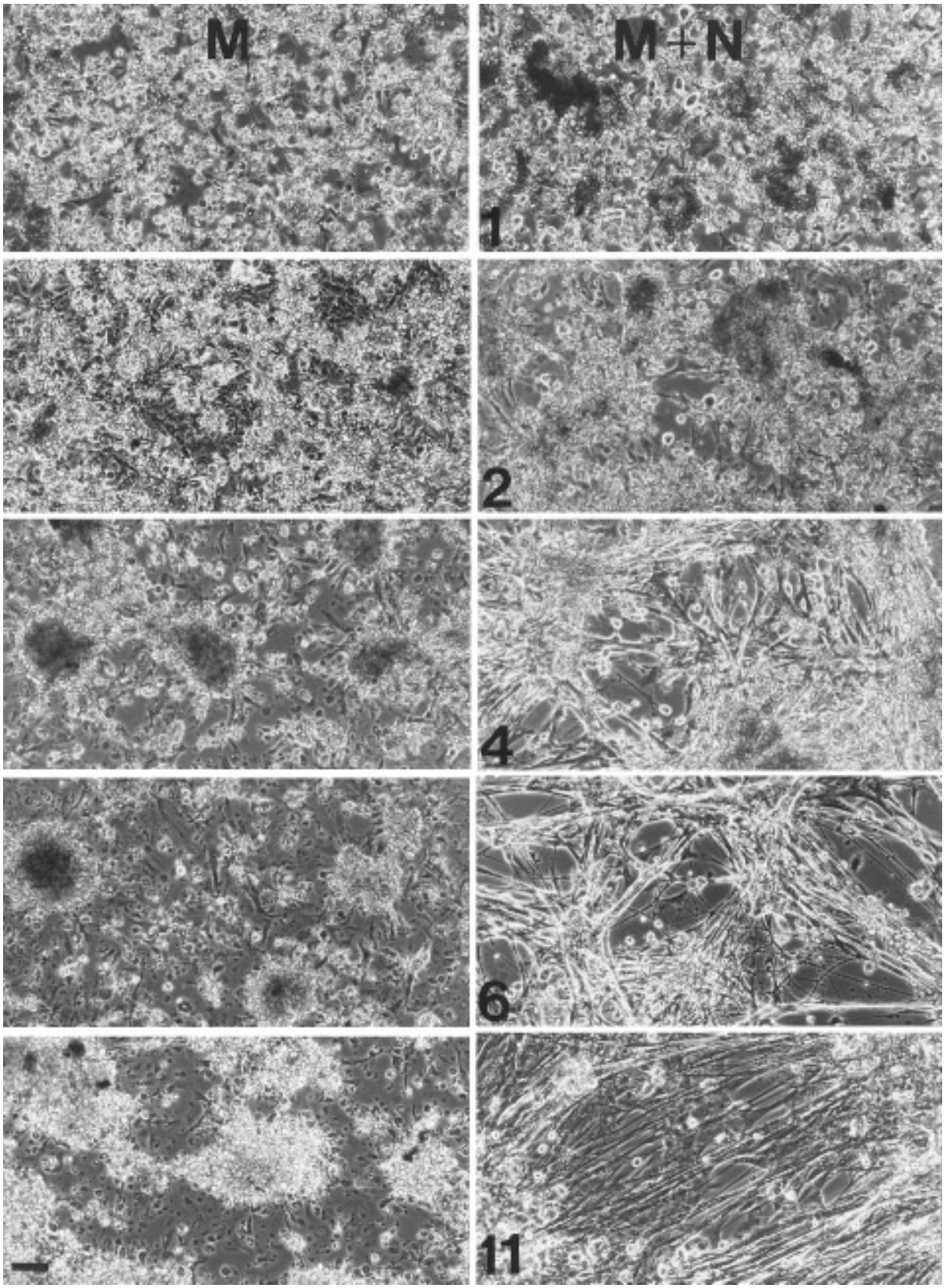
To determine whether the survival of myogenic cells was compromised in the absence of neurons, cell viability was assessed using the LIVE/DEAD cell kit (Molecular Probes; see Materials and Methods). Extensive cell death was not observed in cultures maintained without neurons. In both the presence and absence of neurons the extent of cell death was similar, with fewer than 1% of the cells in the culture dishes dying over the first 4 days *in vitro*. There was no evidence that large numbers of myocytes were dying in the absence of neurons. It remained possible, however, that a small fraction of the total cell population representing the myogenic cells died in the absence of neurons. Arguing against this possibility was the observation that cultures of imaginal leg cells were able to respond to the delayed addition of neurons. Cultures were prepared as above from stage P2 legs and maintained for 7 days in the absence of neurons. As described above, aggregates of round, phase-bright cells formed. On Day 7 neurons were seeded into half of the dishes, which responded with an increase in the number of spindle-shaped cells and the formation of contractile fibers (Fig. 3). Thus, the myogenic cells were able to survive for at least 7 days in the absence of neurons.

### *Neuronal Enhancement of Myogenic Cell Division*

To test the hypothesis that neurons enhance myotube development at least in part by increasing the rate of myogenic cell proliferation, leg cell suspension was seeded into dishes with or without stage P0 neurons that had been plated 2 days earlier. At the desired time point, the cultures were exposed for 8 hr to medium that included 20 $\mu M$ BrdU. After histological processing the number of nuclei that had incorporated BrdU was counted in each dish. In the presence of neurons individual spindle-shaped cells with distinctly labeled nuclei were common, and with longer delays between BrdU exposure and fixation (see below) fibers with multiple labeled nuclei were present (Fig. 4). In an initial experiment, paired cultures were exposed to BrdU 1 day after seeding of the leg cell suspension and then fixed immediately. In cultures that did not contain neurons, a small number of labeled nuclei were found within the cell aggregates, indicating that cells had undergone DNA synthesis *in vitro*. Isolated polygonal flat cells and spindle-shaped cells were occasionally labeled as well.

The number of labeled nuclei was greater in cocultures than in paired dishes that lacked neurons (Fig. 5A). Most of the labeled nuclei in the 1-day-old cocultures were found within the cell aggregates or within groups of spindle-shaped cells. The large nuclei of polygonal flat cells occasionally incorporated BrdU, although these accounted for less than 5% of the labeled nuclei. Label was never detected in neurons in the cocultures and labeled nuclei were never observed in cultures of stage P0 thoracic neurons alone (see also below). To determine the percentage of cells that incorporated BrdU during the 8-hr period, cultures prepared together with those of the experiment described above were

**FIG. 1.** Sequential photographs of a culture dish containing cells isolated from an early pupal (stage P2) developing adult leg (left column; M) and a paired coculture in which cells from the same source were seeded onto a dish containing thoracic neurons from a stage P0 animal that had been plated 2 days earlier (right column; M + N). Numbers refer to the number of days *in vitro*. In the cocultures, cells from the developing leg align and fuse to form contractile myotubes, whereas they remain in large clumps in the absence of neurons. Cal., 100 $\mu$m.



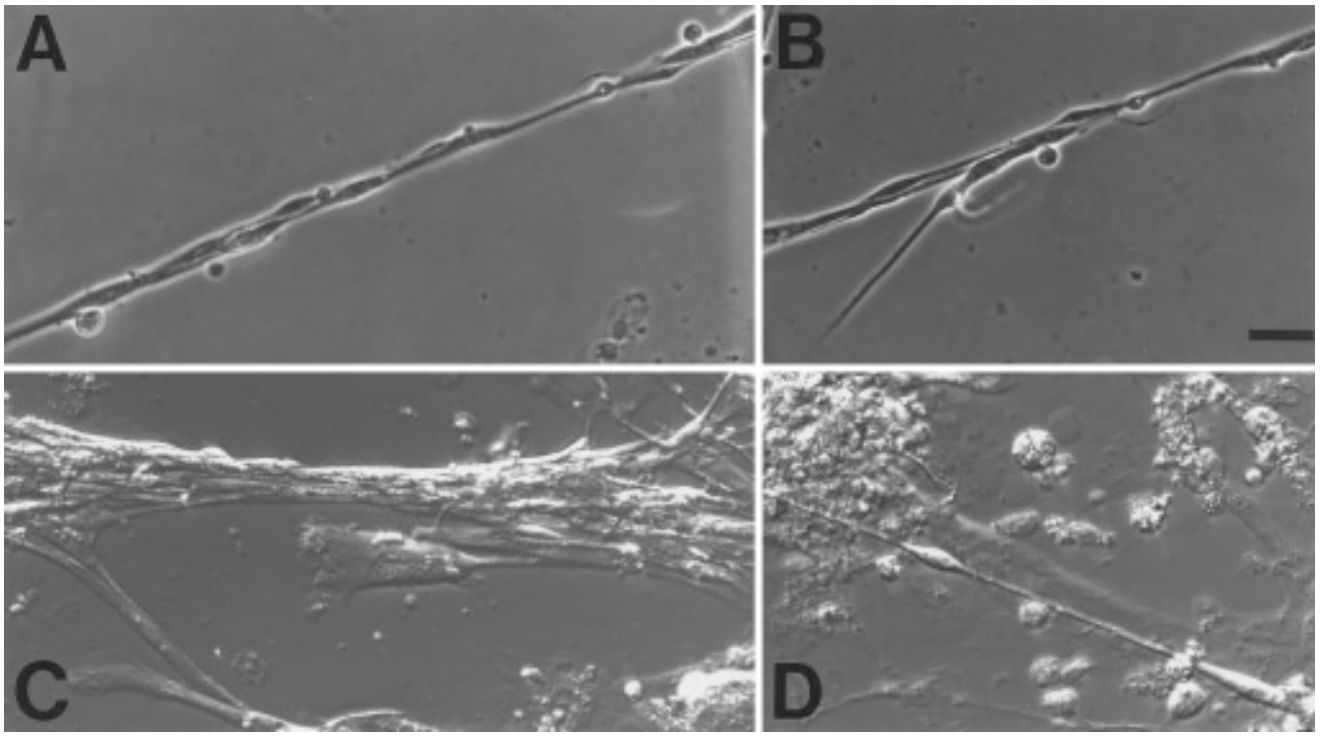



**FIG. 2.** Spindle-shaped cells in a coculture. (A, B) Phase-contrast images of individual spindle-shaped cells aligning and fusing. (C) Hoffmann modulation contrast image of spindle-shaped cells fusing to form a myotube. A neuronal cell body lies in the center of the field. (D) Hoffmann modulation contrast image of two spindle-shaped cells lying end-to-end. Cal., 25 $\mu$m.

stained with propidium iodide and the total number of nonneuronal nuclei counted, yielding 2.8 and 0.8, respectively, as the percentages of nuclei incorporating BrdU with and without neurons.

In separate experiments, paired cultures were exposed to BrdU 2 or 4 days after seeding the leg cell suspension and then fixed immediately. In both cases, the number of labeled nuclei was higher in the dishes containing thoracic neurons (Figs. 5B, 5C). To compare the rates of BrdU incorporation on Days 2 and 4 within the same experiment and, thereby, eliminate interexperimental variability, leg cell suspension was distributed equally among eight dishes, half of which contained thoracic neurons. Two cocultures and two cultures without neurons were fixed after exposure to BrdU for 8 hr 2 days after seeding the leg cell suspension and the other four dishes were exposed to BrdU and fixed after an additional 2 days. At both time points the number of nuclei incorporating BrdU was greater in the dishes containing thoracic neurons (Fig. 5D). Thus, myogenic cells continue to undergo DNA synthesis for at least 4 days after being placed in culture, with the rate of BrdU incorporation being enhanced by the presence of neurons.

To follow the accumulation of labeled nuclei, paired cocultures were exposed continuously to BrdU after seeding the leg cell suspension. Two cultures were fixed at each of six time points within the first 72 hr. The number of labeled nuclei increased at a relatively constant rate over the first 24 hr (Fig. 6A). The rate of accumulation slowed over the subsequent 48 hr, as increasing numbers of labeled nuclei became incorporated into myotubes (see also below).

To determine the fates of cells that underwent DNA synthesis *in vitro,* cell suspension was prepared from stage P2 imaginal legs and divided into 12 culture dishes, half of which contained stage P0 thoracic neurons that had been plated 2 days earlier. One day after seeding the leg cell suspension, the cultures were exposed for 8 hr to medium containing 20 $\mu M$ BrdU, then rinsed in fresh medium and allowed to develop for a further 24, 48, or 72 hr. At all three time points there were more labeled nuclei in the cocultures than in dishes containing myogenic cells without neurons (Figs. 6B, 6C). The number of labeled nuclei did not change markedly with time, suggesting that the nuclei did not divide repeatedly following BrdU incorporation. In all cultures that did not contain neurons, labeled cells were found primarily within the cell aggregates (Fig. 7). Twenty-four hours after BrdU exposure, the labeled nuclei in the cocultures were located within the cell aggregates or within spindle-shaped cells (Fig. 7). At 48 hr, larger numbers of individual spindle-shaped cells were labeled, as well as multiple nuclei within myotubes (Fig. 7). Large numbers of labeled nuclei were located within multinucleate myotubes at 72 hr (Fig. 7). Our interpretation is that cells which undergo DNA synthesis within the aggregates in the young cocultures assume a spindle-like morphology and fuse to generate

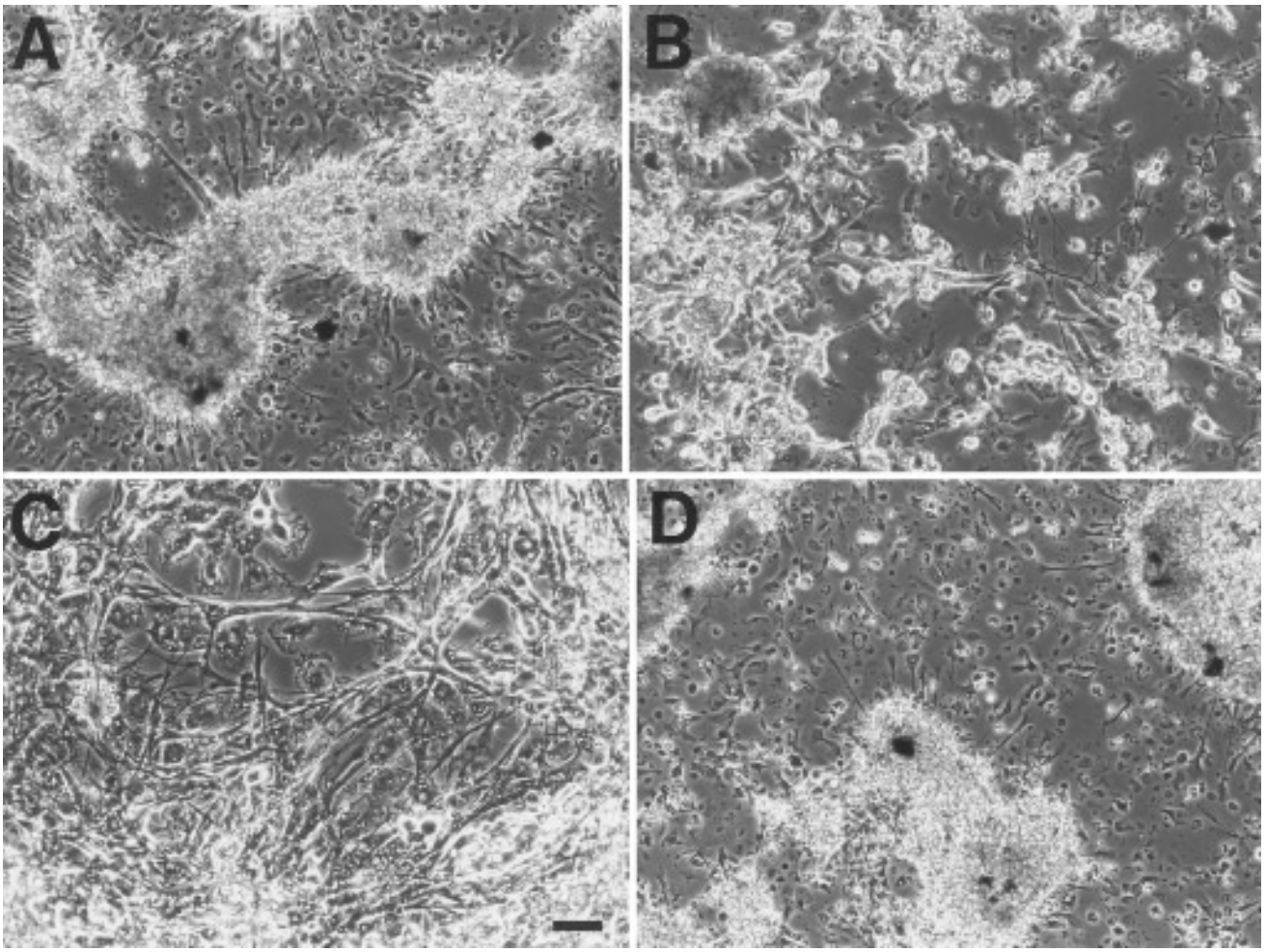



**FIG. 3.** Myogenic cells persist for at least 1 week in culture in the absence of neurons. Cells from a stage P2 developing adult leg were placed in culture. By Day 4 *in vitro* they had aggregated to form large cell clumps (A). On Day 7 *in vitro* neurons dissociated from stage P0 thoracic ganglia were placed in half of the cultures (B; Day 9 *in vitro*). On Day 26 *in vitro* there were numerous spindle-shaped cells and contractile myotubes in the cocultures (C), but not in the dishes to which no neurons had been added (D). Cal., 100 $\mu$m.

myotubes. The number of labeled nuclei was similar at each of the three time points and the labeling was of a relatively uniform dark intensity, suggesting that the cells did not divide repeatedly before differentiation. The successful fusion of labeled nuclei into contractile myotubes also suggests that BrdU incorporation did not hinder the normal development of the myogenic cells as has been noted in other systems (Lough and Bischoff, 1976).

### *Specificity of the Neuronal Effect on Myogenic Cell Proliferation*

The experiments described above reveal that the proliferation of myogenic cells is enhanced in cocultures containing neurons from early pupal thoracic ganglia. These ganglia contain the motoneurons that project to the imaginal leg and innervate the developing muscles (Kent and Levine, 1988; C. Consoulas, K. S. Kent, and R. B. Levine, submitted for publication). To determine whether the ability to enhance myogenic cell proliferation depended upon the presence of these motoneurons, as opposed to neurons in general, paired cultures of myogenic cells were prepared in the presence or the absence of neurons derived from stage P0 brains. Although the stage P0 brain contains large numbers of interneurons, there are few motoneurons, with the exception of those that innervate muscles at the base of the antennae. The effect on the leg cell cultures was similar to that described for thoracic neurons. Within 4 days large numbers of spindle-shaped cells were present in the cocultures and these subsequently fused to form contractile fibers. In a parallel set of experiments brain neurons were found to enhance the level of BrdU incorporation (Fig. 8A). Similar results were obtained with cultures containing neurons from stage P0 abdominal ganglia. Nonneuronal cells

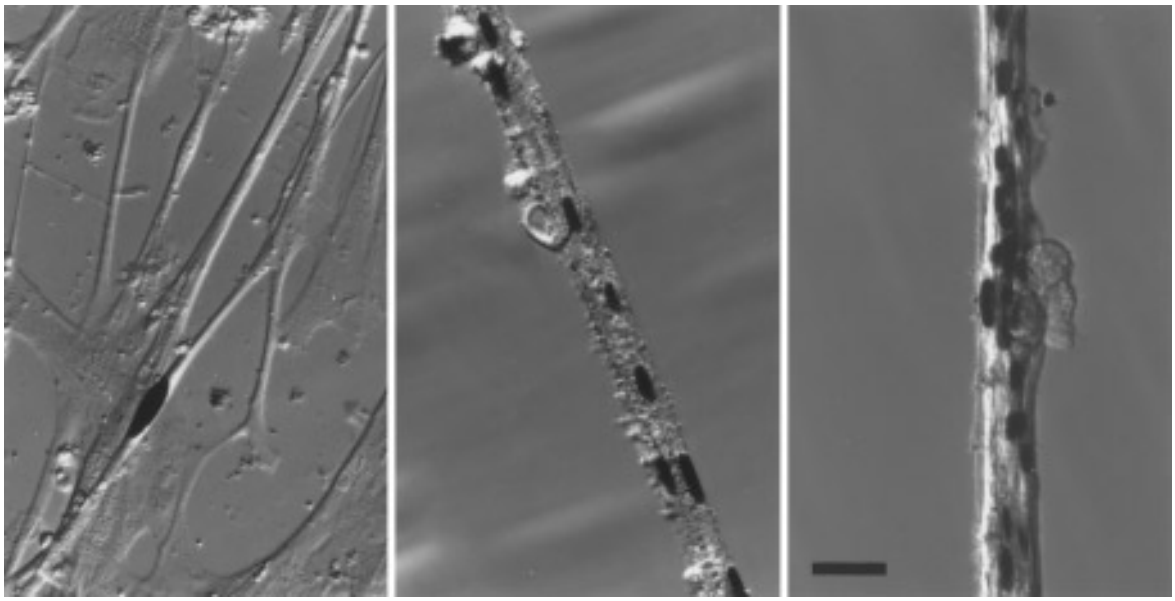


**FIG. 4.** Myogenic cells undergo DNA synthesis *in vitro*. Six-day-old cocultures were exposed for 8 hr to BrdU, then fixed and processed with the antibody to BrdU. Hoffmann modulation contrast images reveal labeled nuclei within an individual spindle-shaped cell (left) and two multinucleate myotubes. Cal., 25 $\mu$m.

derived from the fat body were ineffective in promoting BrdU incorporation or muscle differentiation (not shown).

The thoracic motoneurons that innervate adult leg muscles persist from the larval stage, where they innervate larval leg muscles (Kent and Levine, 1988). To determine whether the ability of these neurons to enhance proliferation was stage-specific, cocultures were prepared using early larval thoracic ganglia as the source of neurons. As with the stage P0 thoracic or brain neurons, the larval thoracic neurons enhanced the level of BrdU incorporation into nuclei (Fig. 8B). Thus, the ability of neurons to enhance the proliferation of myogenic cells was neither stage-specific nor restricted to neurons that would normally innervate the leg muscles.

### *Nature of the Interaction between Neurons and Myogenic Cells*

The neuronal enhancement of myogenic cell division may be mediated by a diffusible signal or may require a more intimate interaction among cells. As initial tests for the presence of a diffusible signal, myogenic cells were maintained in medium that had been ''conditioned'' by prior exposure to either high-density neuronal cultures or whole thoracic ganglia. In neither case was the number of spindle-shaped cells or the level of BrdU incorporation enhanced above cultures maintained in regular medium (not shown). In a further experiment, double-well dishes were prepared in which cells in the two wells were unable to establish physical contacts, but were in communication through the 1 ml of medium in the culture dish. Stage P0 neurons were plated in one well. Two days later cell suspension from stage P2 thoracic legs was seeded into the other well and, in parallel, into single-well dishes with and without neurons. After 2 days the cultures were exposed to 20 $\mu M$ BrdU for 8 hr, then fixed and processed to reveal BrdU incorporation. As in previous experiments, in the single-well dishes, the cocultures contained significantly greater numbers of labeled nuclei than dishes containing leg cell suspension alone (Fig. 9A). In the double-well dishes, no labeled nuclei were observed in the wells containing neurons alone, and in the wells containing myogenic cells the extent of labeling was not significantly different from that in the single-well dishes that did not contain neurons (Fig. 9A).

In a related series of experiments, stage P0 thoracic neurons were plated onto one end of elongated single wells. Two days later cell suspension from stage P2 imaginal legs was seeded onto the center of the dish and the opposite end. Thus, the two cell types were within the same 100 $\mu$l of medium and able to establish physical contacts as neuronal processes grew into the center of the well, but not at either side. After 2 days the dishes were exposed to BrdU for 8 hr, fixed, and processed histochemically. The wells were divided into three regions: the two sides containing neurons or myogenic cells alone and the central region of overlap. The number of labeled nuclei was counted in randomly chosen fields within the three regions. No labeled nuclei were observed in the region containing only neurons and few were located in the region that included only cells from the imaginal leg. By contrast, there were large numbers of labeled nuclei in the regions of overlap, including many spindle-shaped cells and multinucleate myotubes (Figs. 9B

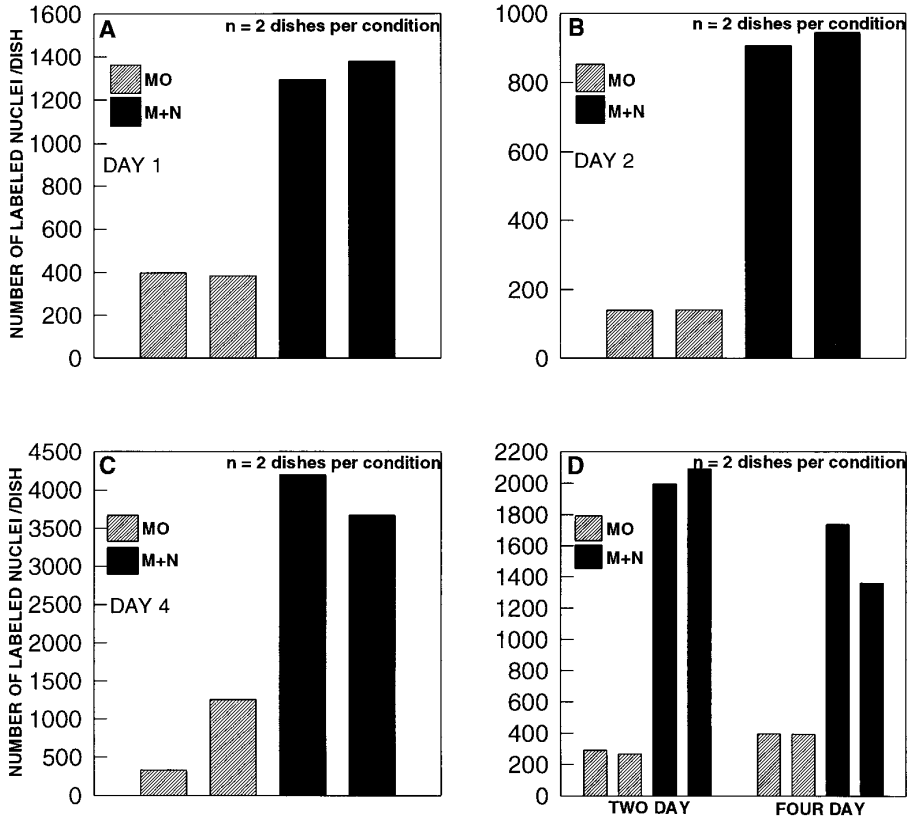



**FIG. 5.** The number of cells undergoing DNA synthesis is enhanced in cocultures. Cells from stage P2 developing adult legs were seeded into culture dishes onto which stage P0 thoracic neurons had been plated 2 days earlier (muscle plus neuron; M + N) and, in parallel, into naive dishes (muscle only; MO). On the day indicated, the dishes were exposed for 8 hr to BrdU, then fixed and processed immunohistochemically to reveal nuclei that had incorporated the thymidine analog. (A) BrdU exposure on the day in which cells from the developing leg were seeded (Day 1). Original density of cells from the developing leg, $2.16 \times 10^4$/dish. The number of labeled nuclei in the cocultures is approximately three times higher than in the dishes not containing neurons. (B) BrdU exposure on the day following seeding of the cells from the developing leg (Day 2). Original cell density, $5 \times 10^4$/dish. (C) BrdU exposure on Day 4. Original density, $5.56 \times 10^4$/dish. (D) Cells from stage P2 developing adult leg were seeded into eight dishes, four of which already contained neurons. Half of the dishes were exposed to BrdU on Day 2 and half on Day 4. Original cell density, $3.28 \times 10^4$/dish. Note that the level of BrdU incorporation varies among experiments (e.g., compare A, B, and C), but that within an experiment the number of labeled nuclei is consistent among dishes and is increased in the presence of neurons.

and 10). The number of labeled nuclei was significantly higher in the region of overlap (Kruskal–Wallis one-way ANOVA $P < 0.01$, with Dunn's posthoc comparison, $P < 0.05$). Thus, the neuronal enhancement of myogenic cell proliferation requires either direct contact or a close-range influence.

### *Requirement for Live Neurons*

To determine whether live neurons are required for the enhancement of myogenic cell proliferation, stage P0 thoracic neurons were plated as above, then fixed 2 days later by a 10-min exposure to acidified alcohol or 3% paraformaldehyde. After extensive rinsing, leg cell suspension was seeded over the neurons. Although the cells aggregated normally and remained viable for at least a week, relatively few spindle-shaped cells appeared in these dishes and no contractile fibers formed. Moreover, fixed neurons did not enhance BrdU incorporation, as assessed in a similar experiment in which the cultures were exposed to BrdU 2 days after seeding the leg cell suspension (Fig. 11A).

To test the hypothesis that neurons "condition" the substrate in a manner that promotes BrdU incorporation into muscle precursor cells, 2-day-old cultures of thoracic neurons were exposed to distilled $H_2O$ for 5 min, causing the neurons to lyse and, in most cases, to lift off the culture dish. Leg cell suspension was seeded onto these dishes and, in parallel, onto dishes that had been similarly treated with water but had never contained neurons, and dishes containing normal 2-day-old neuronal cultures. Two days later all dishes were exposed for 8 hr to BrdU. As before, the number of labeled nuclei was higher in the cocultures con-

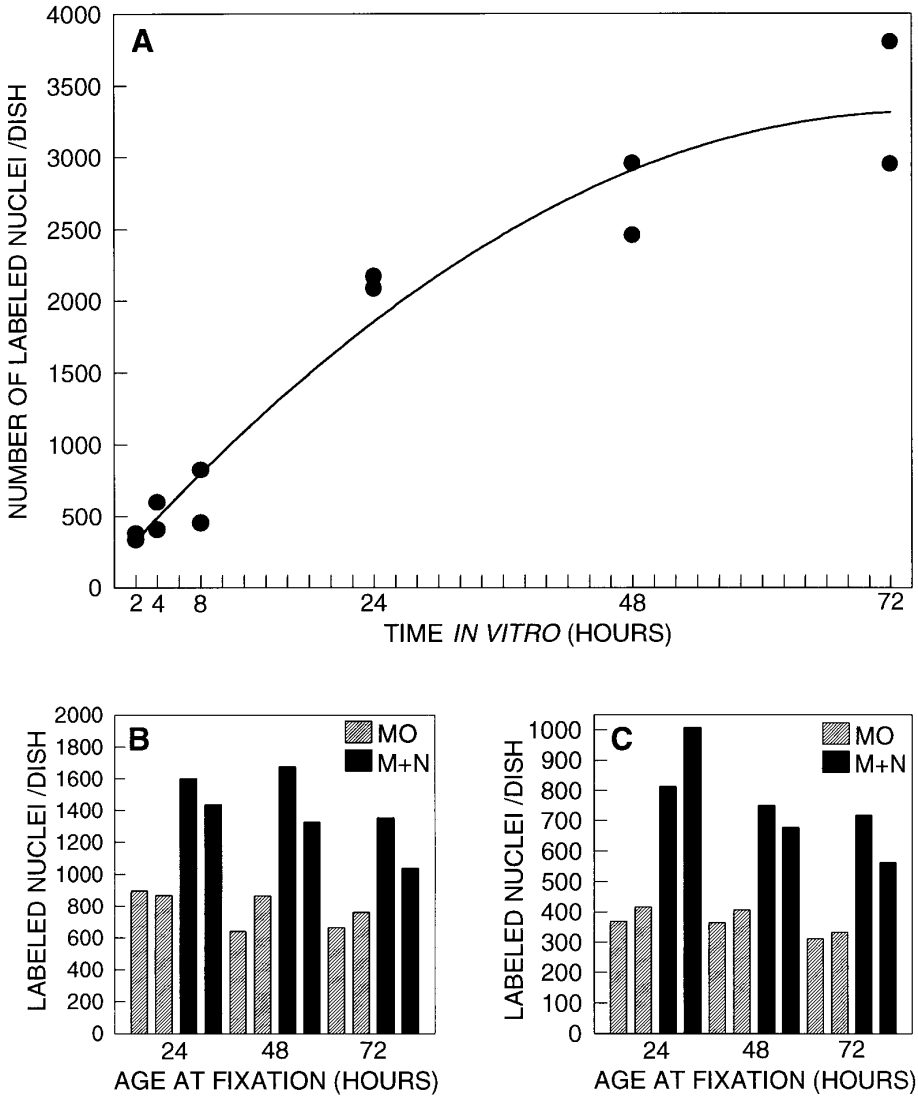



**FIG. 6.** Time course of nuclear labeling. (A) Continuous exposure to BrdU. Twelve replicate cocultures were maintained continuously in medium containing BrdU. Two dishes were fixed at 2, 4, 8, 24, 48, and 72 hr. The rate of increase in the number of labeled nuclei is relatively constant over the first 24 hr, then it slows. (B, C) Pulse-labeling experiment. Cells from stage P2 developing adult leg were seeded into 12 dishes, half containing 2-day-old cultures of thoracic leg neurons. One day later, the cultures were exposed for 8 hr to BrdU. Four of the dishes were fixed immediately (24 hr, MO and M + N); 2 MO and 2 M + N dishes were then fixed at 48 and 72 hr. Note that at each time point there were more labeled nuclei in the cocultures. Original cell density: (A) $6.16 \times 10^4$ cells per dish; (B) $8.56 \times 10^4$ cells per dish; (C) $4.36 \times 10^4$ cells per dish.

taining live neurons. The number of labeled nuclei was not significantly different, however, between in the distilled water-treated dishes that had or had not contained neurons (Fig. 11B).

### *Role of Neuronal Activity*

To determine whether the enhancement of myogenic cell division depends upon neuronal activity, cocultures were prepared as above, but maintained in the presence of $10^{-6}$ *M* TTX. Tetrodotoxin at this level blocks $Na^+$ currents irreversibly in cultured *Manduca* neurons (Hayashi and Levine, 1992) or *in vivo* (Trimmer and Weeks, 1993). By contrast, TTX-sensitive currents have not been reported in insect muscle cells (Salkoff and Wyman, 1983). Continuous exposure to TTX did not affect cell proliferation or the differentiation of contractile fibers. The number of nuclei incorporating BrdU was similar in cocultures with or without TTX and cocultures with TTX had significantly higher numbers of labeled nuclei than cultures maintained without neurons (one-way ANOVA $P < 0.01$, with post hoc comparison, Sheffe $F$ test $P < 0.05$; Fig. 12). Thus, $Na^+$-based action

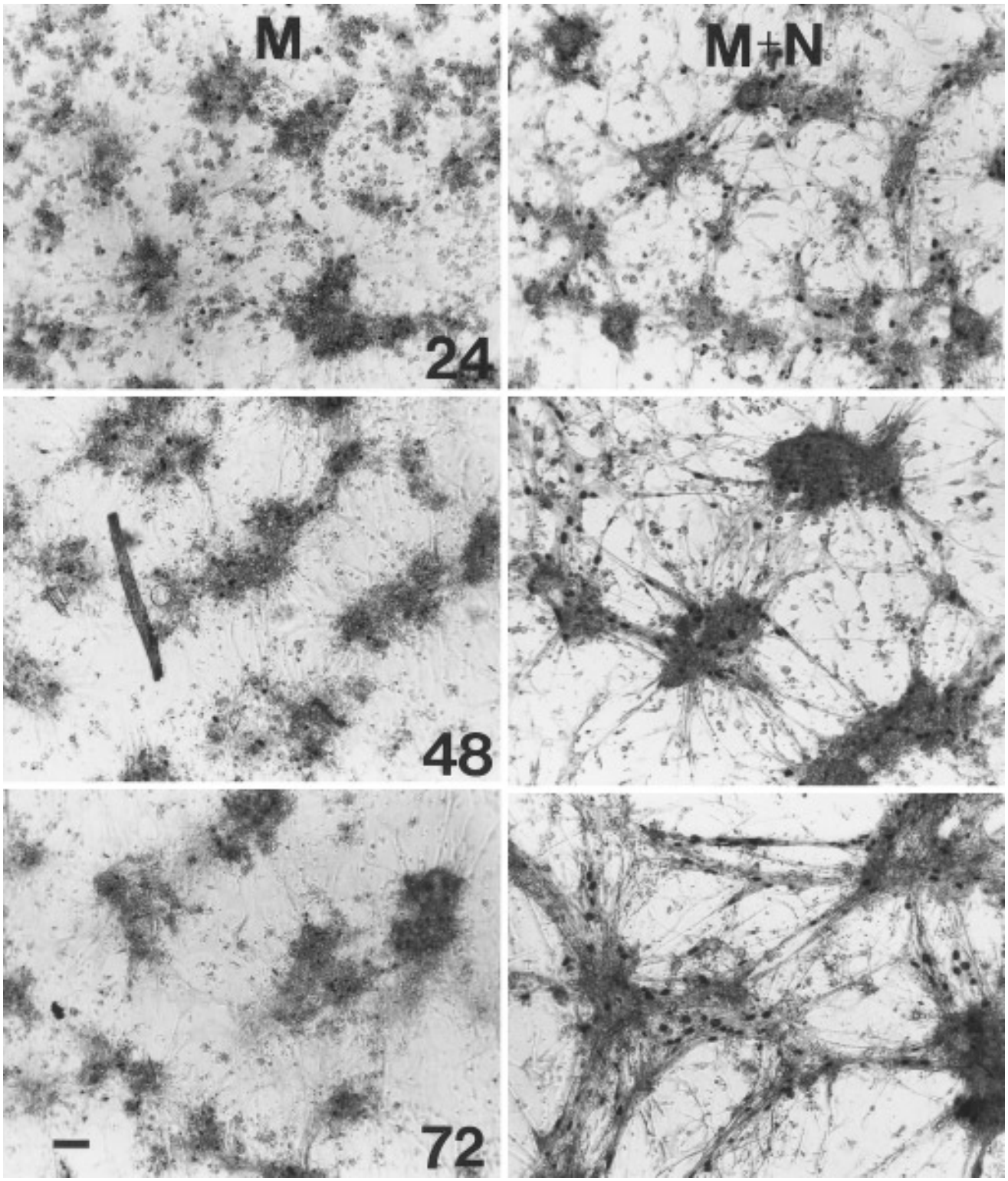



**FIG. 7.** Same experiment as in Fig. 6B. Bright-field photographs showing cells isolated from stage P2 developing adult legs and plated alone (M) or in dishes already containing cultures of stage P0 thoracic neurons (M + N). Dishes were exposed for 8 hr to BrdU 1 day after plating and fixed immediately thereafter (24 hr) or on the following days (48 and 72 hr). Note that there are more labeled nuclei in the cocultures (M + N) at each time point. The cells incorporating BrdU were found mainly within cell clusters at 24 hr; some are found within individual spindle-shaped cells that have moved away from the clusters at 48 hr, and many are located within multinucleate myotubes by 72 hr. Cal., 100 $\mu$m.

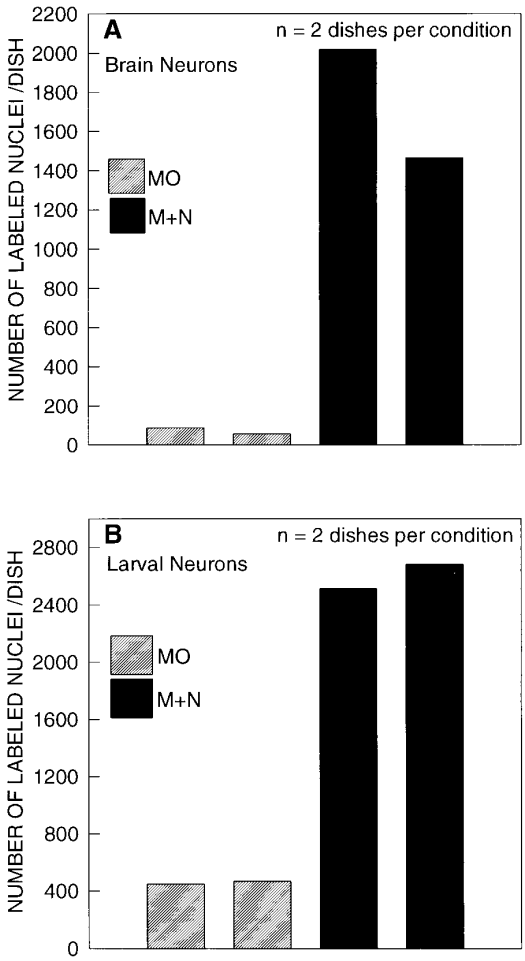



**FIG. 8.** Neuronal enhancement of myogenic cell proliferation is not unique to pupal thoracic leg motoneurons. (A) Cells from stage P2 developing adult legs were seeded into four culture dishes, half of which already contained neurons derived from a stage P0 brain. The cultures were exposed for 8 hr to BrdU on the next day and then fixed immediately. Note that the number of labeled nuclei is greater in the cocultures (M + N). Original seeding density: 3.82 $\times 10^4$ cells per dish. (B) Same experimental protocol, but neurons were derived from early larval (stage L2) thoracic ganglia. Again, the number of labeled nuclei is higher in the cocultures. Original seeding density: 6.88 $\times 10^4$ cells per dish.

potentials are not necessary for the neuronal effect on muscle precursor cells.

### *Effect of 20-Hydroxyecdysone on Myogenic Cell Division*

Metamorphosis is initiated in response to elevations in the hemolymph level of the steroid hormone 20-HE, which regulates many of the neuronal changes that occur during adult development (Weeks and Levine, 1990; Levine *et al.*, 1991, 1995). The level of 20-HE increases during the first days of adult development (Bollenbacher *et al.*, 1981), coincident with the onset of intense myoblast proliferative activity within the imaginal legs (C. Consoulas, K. S. Kent, M. Anezaki, and R. B. Levine, submitted for publication). The experiments described to this point were all carried out in the presence of physiological levels of 20-HE. To determine whether 20-HE influenced the rate of myogenic cell proliferation, stage P0 neuronal cultures were maintained for 2 days with normal levels of 20-HE (1 $\mu$g/ml) and then seeded with leg cell suspension. Half of these cultures were maintained in 1 $\mu$g/ml 20-HE, while the other half were maintained in medium lacking 20-HE. Cultures containing leg-cell suspension only were prepared in parallel. After 2 days, the cultures were exposed for 8 hr to BrdU and fixed. Counts of labeled nuclei revealed a significantly higher level of BrdU incorporation in the cultures maintained with 20-HE (Fig. 13; Kruskal–Wallis one-way ANOVA $P < 0.01$, with Dunn's post hoc comparisons $P < 0.05$), suggesting that myogenic cell proliferation was enhanced by the steroid hormone. Even in the absence of neurons the level of BrdU incorporation was greater in the presence of 20-HE, suggesting independent neuronal and hormonal effects.

## DISCUSSION

### *Characteristics of Muscle Precursor Cells Derived from the Imaginal Legs*

The myocytes derived from the imaginal leg of *Manduca* are similar in appearance to those described in other insect culture systems. Cultures prepared from *Drosophila* (Seecof *et al.*, 1973) or cockroach (Bermudez *et al.*, 1986) embryos contain spindle-shaped cells that align, fuse, and differentiate into contractile fibers. Although we have yet to study the further differentiation of these fibers in detail, those in cockroach cultures become innervated and express sarcomerically arranged contractile elements (Bermudez *et al.*, 1986). The spindle-shaped muscle precursors in these cultures are also similar in appearance to those present in vertebrate muscle cultures (e.g., Bischoff and Holtzer, 1969; Buckley and Konigsberg, 1974; Konigsberg, 1963).

At early time points following BrdU exposure, labeling occurs extensively within the aggregates of round phase-bright cells, whereas with longer post-BrdU chase periods more labeled nuclei appear first within spindle-shaped cells and then within multinucleate myotubes. It seems likely, therefore, that cells within these aggregates represent the mitotic myogenic cells (replicating myoblasts), the progeny of which assume a spindle-like morphology while migrating away from the aggregates. The shape and fate of mammalian muscle precursor cells are influenced by the substrate (Ocalan *et al.*, 1988), suggesting that the laminin/ConA substrate used in the present experiments may similarly influence the muscle precursors as they migrate away from the cell aggregates and onto the dish surface. We refer to the spindle-shaped cells as myocytes because they clearly fuse

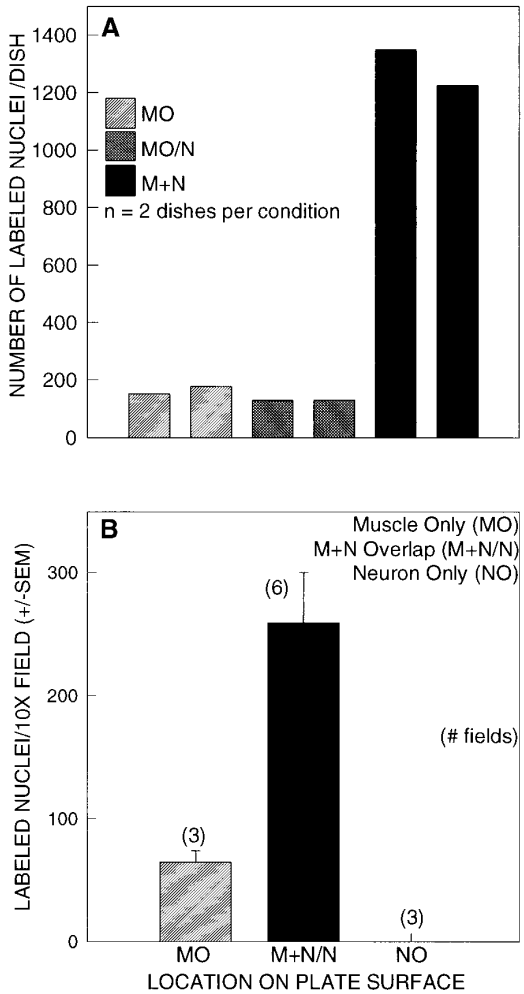



**FIG. 9.** The mitogenic influence of neurons on myogenic cells is probably not mediated by an abundant, freely diffusible molecule. (A) Cells from stage P2 developing adult legs were divided and seeded into dishes containing no neurons (MO), dishes into which stage P0 thoracic neurons had been plated 2 days previously (M + N), or one well of a double-well dish. In the latter case, stage P0 thoracic neurons had been plated in the other well 2 days previously (MO/N). The medium (1 ml) was not changed and was allowed to diffuse freely between the two wells (which were separated by 3 mm), but the neurons and myogenic cells could not contact one another. After 2 days the dishes were exposed for 8 hr to BrdU, then fixed and processed immunohistochemically. Note that the number of nuclei incorporating BrdU was higher in the cocultures (M + N), but was not enhanced in the double-well dishes (MO/N). There were no labeled nuclei in the well containing neurons only (not shown). (B) Related experiment in which neurons were plated on one side of an elongated well containing 100 $\mu$l of medium. After 2 days myogenic cells were seeded onto the center and the opposite end of the well and maintained in the same 100 $\mu$l of medium. Two days later the dish was exposed for 8 hr to BrdU, then fixed and processed immunohistochemically. The well was divided into three regions: the extreme side onto which the myogenic cells were plated (MO), the extreme side onto which the neurons were plated (NO), and the middle region where myogenic cells overlapped with neuronal processes and some cell bodies (M + N overlap; M + N/N). The number of labeled nuclei was counted in randomly chosen fields within the three regions. Note that there were more labeled nuclei per field in the center region where neurons and myogenic cells overlapped. There were no labeled nuclei in the fields containing only neurons.

into multinucleate fibers that become contractile. It is not clear, however, whether or not these cells are postmitotic. Although large numbers of spindle-shaped cells are born *in vitro*, as indicated by the nuclear labeling following BrdU exposure, we have no evidence that these cells go through subsequent rounds of DNA synthesis before differentiation. Indeed, with rare exceptions, the BrdU labeling of nuclei within the spindle-shaped cells and multinucleate fibers is uniformly dark, suggesting that the spindle-shaped cells have completed a terminal mitosis before fusion (Bischoff and Holtzer, 1969) and that, as in vertebrate muscle cultures (Konigsberg, 1963), myonuclei do not divide after fusion.

In vertebrate myoblast cultures, the decision of whether to divide or differentiate is influenced markedly by levels of serum or growth factors in the medium (Clegg *et al.*, 1987; Cusella-De Angelis *et al.*, 1994; Olson, 1992; Olsen *et al.*, 1986; Templeton and Hauschka, 1992). We have yet to examine this issue, but under the culture conditions employed in this study, replicating myogenic precursors were present for at least 1 week, as assessed with BrdU incorporation, and continued to incorporate label even as other cells were differentiating into contractile fibers.

### *Neuronal Effects on Muscle Fiber Formation*

The level of myogenic cell division, as indicated by the number of nuclei incorporating BrdU during an 8-hr period, is enhanced significantly by the presence of neurons in cocultures. In parallel, there is an increase in the number of spindle-shaped myocytes present in the cultures and enhanced formation of multinucleate contractile fibers. It is not clear whether the enhanced formation of contractile fibers is due solely to the increased number of myocytes or whether neurons also promote the differentiation of muscle fibers. Neurons are not critical for the early phases of muscle differentiation since in cultures that do not contain neurons a limited number of myocytes is present and the myocytes sometimes fuse. Moreover, it is unlikely that neurons are necessary for the survival of myogenic cells since the level of cell death was not higher in the absence of neurons and neurons were still able to promote the formation of contractile fibers when their addition to cultures was delayed by 1 week. In older cocultures, contractile fibers persisted long after all of the neurons had died.

The ability of neurons to enhance myogenic cell proliferation is not absolutely specific, in that neurons other than the pupal motoneurons that normally innervate developing adult leg muscles had a comparable effect. Thus neurons from abdominal ganglia, brain, or larval thoracic ganglia caused increased proliferation of myogenic cells and en-

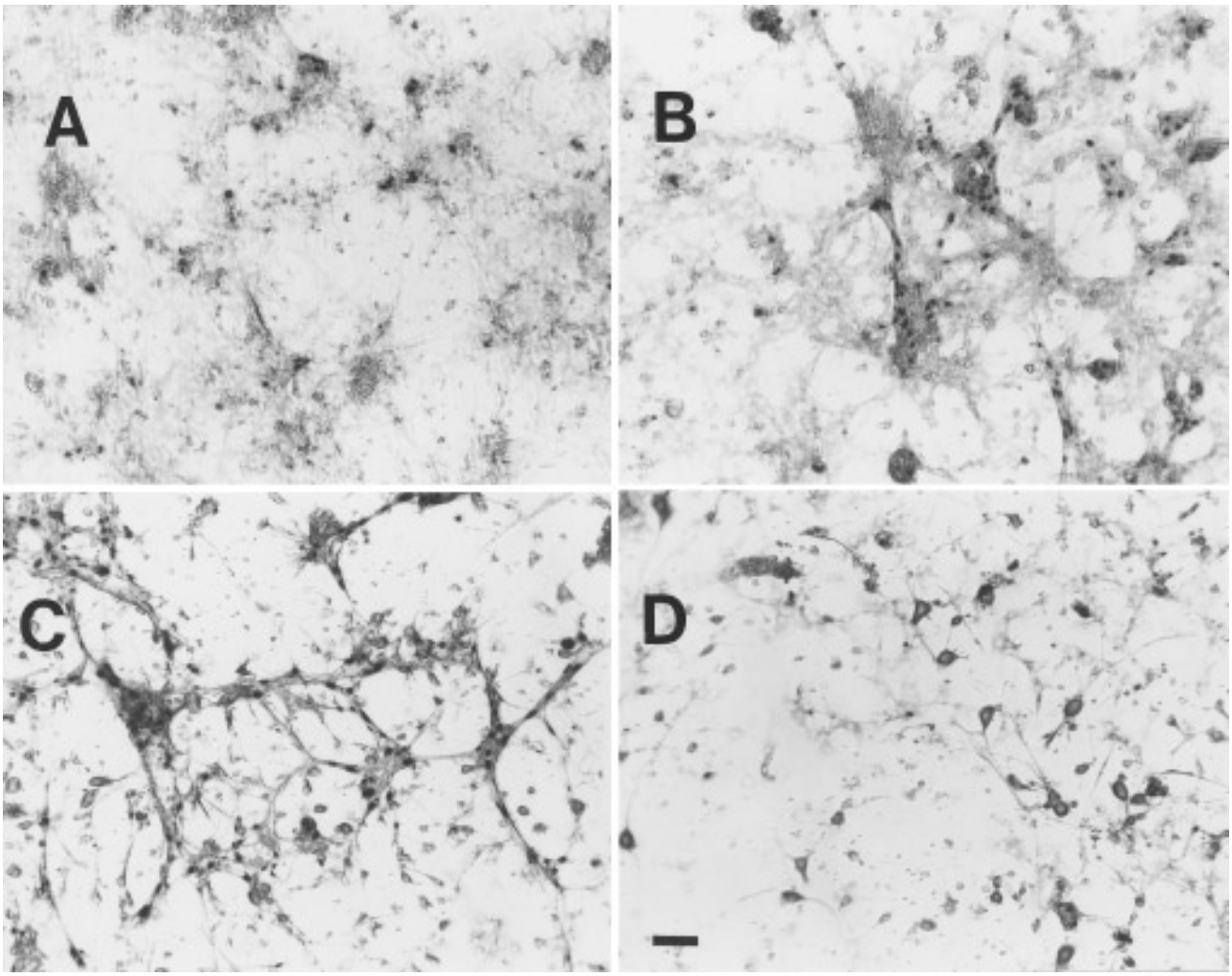



**FIG. 10.** Bright-field photographs of the same experiment as in Fig. 9B. (A) Region containing only imaginal leg cells. Note that there are few labeled nuclei. (B and C) Two regions nearer to the center of the well, with C being further to the side containing neurons. Note that there are many BrdU-labeled nuclei. (D) Region of the well containing only neurons. There are no labeled nuclei. Cal., 100 $\mu$m.

hanced formation of contractile fibers. Nonneuronal tissues, however, could not mimic this effect.

The mitogenic influence provided by neurons is unlikely to involve a long-lived, widely diffusible molecule. Attempts to ''condition'' medium by allowing dense neuronal cultures to develop in small volumes were unsuccessful, nor was proliferation enhanced when whole thoracic ganglia were included within the cultures. Similarly, proliferation was not enhanced in double-well experiments in which contact between neurons and muscle precursor cells was blocked, but both cell types were exposed to the same restricted volume of medium. In cocultures prepared in elongated wells, enhanced incorporation of BrdU was restricted to regions of neuron/myogenic cell overlap. Despite these findings, a neuronally derived diffusible factor may be present at extremely low levels or may be degraded rapidly under our culture conditions.

Fixed neurons did not enhance BrdU incorporation or myogenic cell proliferation. This suggests that membrane-bound molecules on the neuronal surface or molecules secreted into the extracellular matrix by neurons are not sufficient, although fixation may have disrupted critical components. Similarly, following removal of the neurons with distilled water, BrdU incorporation was not enhanced, suggesting either that the neurons do not condition the substrate or that the conditioning involves a water-soluble factor.

Tetrodotoxin, which blocks sodium-based neuronal action potentials in this system (Hayashi and Levine, 1992), did not prevent the enhancement of myogenic cell division. The possibility remains, however, that forms of neuronal activity that do not require sodium-dependent action potentials, for example voltage-gated $Ca^{2+}$ influx, may be important.

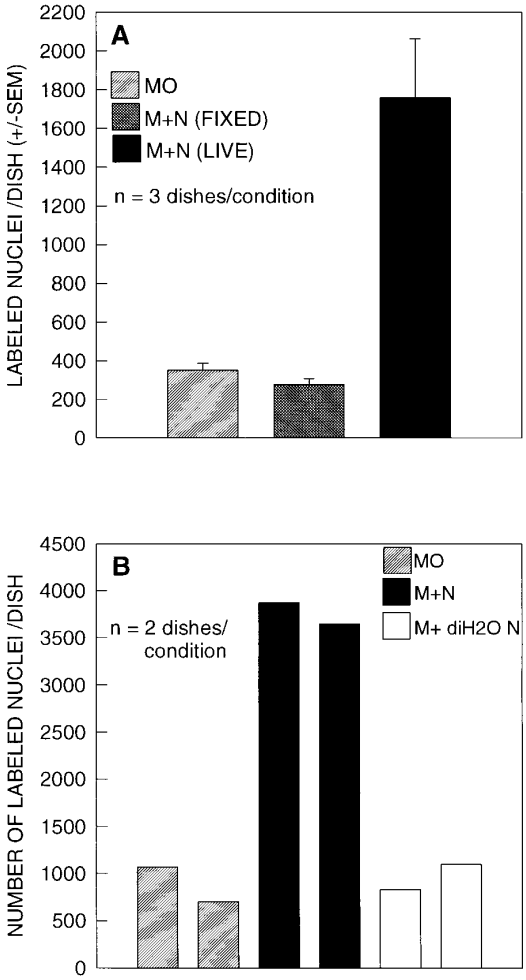



**FIG. 11.** (A) Gentle fixation (3% paraformaldehyde for 10 min) destroys the ability of neurons to promote nuclear division. Myogenic cells were seeded onto 12 dishes, either alone, with live neurons, or onto dishes containing fixed neurons. Original cell density: $7.12 \times 10^4$ cells per dish. (B) Similar experiment, but myogenic cells were seeded onto neuronal cultures that had been treated for 5 min with distilled $H_2O$. Original cell density: $5.68 \times 10^4$ cells per dish.

### *Role of 20-Hydroxyecdysone*

In nerve/muscle cocultures the level of myogenic cell proliferation was enhanced by 20-HE. This reflects, at least to some degree, a direct action of the steroid hormone on cells derived from the imaginal legs, since 20-HE increased the number of BrdU-labeled nuclei in cultures that did not contain neurons. In addition, 20-HE may act via the neurons, by promoting the expression or release of a mitogenic factor. Another possibility, given the ability of 20-HE to enhance the growth of cultured pupal neurons (Prugh *et al.*, 1992), is that there are more chances for nerve/muscle interaction due to the greater extent of neuronal arborizations in the presence of 20-HE. One argument against the latter possibility is that the neuronal cultures were exposed uniformly to 20-HE for 2 days prior to the addition of myogenic cells. The extent of neuronal branching was unlikely to be significantly different under the two hormonal conditions 2 days later when BrdU incorporation was monitored (Prugh *et al.*, 1992). An ecdysteroid influence on myogenic cell proliferation *in vivo* has been observed in studies of postembryonically developing abdominal muscles in *Manduca* (Hegstrom and Truman, 1996). Furthermore, elevations in the ecdysteroid titer *in vivo* (Bollenbacher *et al.*, 1981) correspond with periods of intense myoblast proliferation within the developing adult legs (C. Consoulas, K. S. Kent, M. Anezaki, and R. B. Levine, submitted for publication).

### *Nature of the Mitogenic Interaction between Neurons and Myogenic Cells*

On the basis of these coculture experiments we conclude that the failure of denervated imaginal muscle to develop *in vivo* reflects, at least in part, a neuronal enhancement of myoblast proliferation. Although the mechanism underlying this mitogenic interaction remains unclear, our observations suggest five alternative hypotheses, which are not mutually exclusive. The first possibility is that neurons must

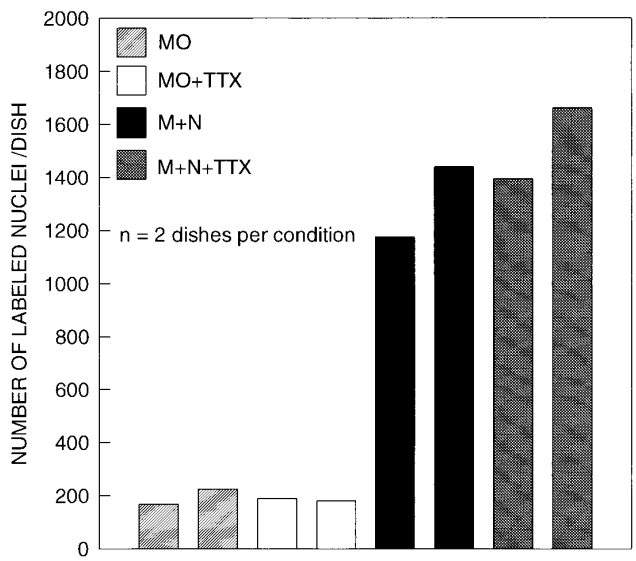



**FIG. 12.** Neuronal activity is not required for the enhancement of myogenic cell proliferation. Cells from stage P2 developing adult legs were divided and seeded into eight dishes, four of which contained stage P0 neurons that had been plated 2 days previously. Half of the cultures were maintained in medium containing $10^{-6}$ *M* TTX, which is sufficient to block $Na^+$ current-dependent action potentials in the neurons. After 2 days the cultures were exposed for 8 hr to BrdU, then fixed and processed immunohistochemically. Note that TTX did not influence the number of labeled nuclei, nor did it block the enhanced BrdU incorporation in cocultures. Original cell density: $4.72 \times 10^4$ cells per dish.

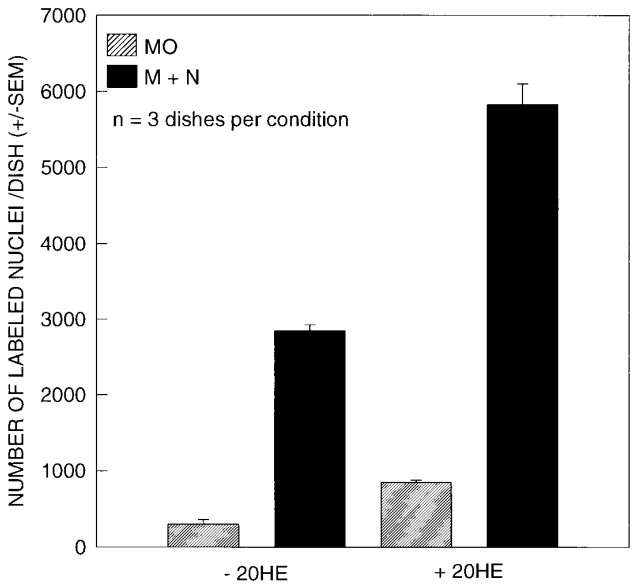



**FIG. 13.** 20-Hydroxyecdysone enhances the incorporation of BrdU. Cells from stage P2 developing adult legs were divided among 12 dishes, half of which already contained stage P0 thoracic neurons. Half of these dishes were maintained in medium containing 1 $\mu$g/ml 20-HE and the other half in medium lacking ecdysteroids. After 2 days the dishes were exposed for 8 hr to BrdU, then fixed and processed. Note that 20-HE increased the number of nuclei incorporating BrdU both with and without neurons, and that neurons were able to enhance BrdU incorporation into the nuclei of myogenic cells even in the absence of ecdysteroids.

contact the myogenic cells directly to promote their division. Neurons ramify extensively within leg cell aggregates in the cocultures, as revealed following neuronal staining (R. Luedeman and R. B. Levine, unpublished observations), but further work is necessary to determine whether intimate contacts or specialized junctions occur, and whether they are critical. Such an interaction is likely to require active transduction of external signals or signal production by the neurons because fixed neurons did not enhance BrdU incorporation or promote muscle fiber formation. Direct nerve–myoblast contacts would be possible *in vivo*. Following the degeneration of larval muscles, the motor terminals remain in the periphery in close association with the adult muscle anlagen (Stocker and Nüesch, 1975; Truman and Reiss, 1995; C. Consoulas, K. S. Kent, and R. B. Levine, submitted for publication). A second possibility is that neurons promote aggregation of the myogenic cells, which in turn leads to enhanced cell division. Indeed, there is direct evidence in *Drosophila* for migration along motor axons and accumulation of muscle precursor cells at nerve terminals (Currie and Bate, 1991; Fernandes and VijayRaghavan, 1993). Similarly, in the imaginal legs of *Manduca,* muscle precursor cells accumulate near motoneuron terminals (C. Consoulas, K. S. Kent, M. Anezaki, and R. B. Levine, submitted for publication). We have not yet determined whether myogenic cells accumulate preferentially along neuronal processes in the cocultures, but one argument against the possibility that such accumulation promotes cell division is that these cells are already highly aggregated in the absence of neurons. The third possibility is that neurons release into the medium a rapidly degraded mitogen or one that is present at such low levels that it cannot be detected in conditioned medium bioassays. Insect motoneurons are known to express peptide cotransmitters (O'Shea and Adams, 1981) or growth factors (Gorczyca *et al.*, 1993), which may exert a mitogenic influence. A fourth possibility is that neurons secrete a mitogen that binds to components of the extracellular matrix (Rapraeger *et al.*, 1991) or a factor that influences the adhesion of myoblasts to the substrate and their subsequent development (Ocalan *et al.*, 1988). One argument against this possibility is that the enhancement of BrdU incorporation was prevented by removing neurons from the substrate before seeding muscle precursors into the culture dishes. This hypothesis deserves further consideration, however, since distilled water may have compromised extracellular components. A fifth possibility is that the myogenic cells themselves, or other cell types from the imaginal leg, are induced to release a mitogen when in the close presence of neurons. The neuronal cultures no longer contain glial cells after 2 days *in vitro* (Prugh *et al.*, 1992), but other cell types are present in low numbers in the tissue derived from the imaginal legs.

The results obtained in the culture system support the hypothesis that motor neurons normally play a role in the regulation of myoblast proliferation during metamorphosis. An alternative interpretation, however, is that in culture the neurons provide a signal that is normally derived from another cell type *in vivo*. Epidermal cells, for example, provide cues that are essential for normal muscle migration, fusion, and attachment (Volk and VijayRaghavan, 1994). Although neuronal influences may not be the only factors that influence muscle development, the consequences of denervation during metamorphosis *in vivo* support the hypothesis that they play an important role. Denervation prior to the onset of metamorphosis compromises or prevents the formation of new adult muscles in lepidoptera (Nüesch, 1985; Thorn and Truman, 1989; Kent *et al.*, 1995; Hegstrom and Truman, 1996; R. Bayline, A. B. Khoo, and R. Booker, submitted for publication; C. Consoulas and R. B. Levine, unpublished observations) and *Drosophila* (Currie and Bate, 1995). This effect is due, at least in part, to a reduction in the rate of myoblast proliferation (Nüesch, 1985; Kent *et al.*, 1995; Hegstrom and Truman, 1996; R. Bayline, A. B. Khoo, and R. Booker, submitted for publication; C. Consoulas and R. B. Levine, unpublished observations), although additional affects on myoblast accumulation and fiber growth remain possible.

Neural interactions regulate the proliferation of muscle (Ontell *et al.*, 1992), glial (Ratner *et al.*, 1988), and neuronal (Selleck *et al.*, 1992) precursors. The insect neuromuscular system may provide a useful model for further investigation of the molecular basis for such interactions, which provide an important mechanism for the coordinated regulation of development in different cell types. *Drosophila* mutants

have been described in which critical elements of putative transduction pathways within the neurons or muscle precursors are disrupted (Fernandes and Keshishian, 1995), and similar tools are available for exploring cell cycle regulation (Selleck *et al.,* 1992) and hormone transduction pathways (Levine *et al.,* 1995). In parallel, the larger size of *Manduca* facilitates our ability to perform surgical and endocrine manipulations *in vivo,* whereas the coculture system provides a useful model for testing mechanistic hypotheses about the influence of cell interactions and steroid hormones on muscle development.

## ACKNOWLEDGMENTS

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